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A Mutation in Bovine Keratin 5 Causing Epidermolysis Bullosa Simplex, Transmitted by a Mosaic Sire

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A mechanobullous skin disorder was identified in the progeny of a 3-y-old Friesian–Jersey crossbred bull. The condition presented as loss of skin and mucosa from contact areas and inflammation. Examination of skin samples under light microscopy revealed separation of the epidermis from the dermis. Electron microscopic analysis refined the site of cleavage to above the basement membrane involving lysis of basal keratinocytes. These observations were consistent with the simplex form of epidermolysis bullosa (EB) in humans. Candidate genes based on human gene mutations were assessed, resulting in keratin 5 being identified as the most likely candidate gene. The sequence of bovine keratin 5 was established and sequencing led to identification of a G to A substitution in all affected animals. This mutation leads to an amino acid change of glutamic acid to lysine in the final E (478) of the KLLEGE motif of the protein. The sire carried a *de novo* mutation and was mosaic, explaining his asymptomatic status and the less than expected frequency of affected offspring. Remarkably, the same mutation has been previously described in EB simplex in humans.

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A dairy industry breeding company in New Zealand (Hamilton, Livestock Improvement Corporation) operates a sire-testing scheme to identify bulls of high genetic merit. Around 300 yearling bulls enter the scheme each year, derived from matings between elite bulls and high genetic merit cows. These include approximately 60 bulls that are termed Kiwicross (predominantly Friesian/Jersey crossbreds). A minimum of 70 daughters per yearling bull are farmed within the 350–400 herds throughout the country that participate in the testing scheme. During the 2002 calving season a mechanobullous skin condition in a number of newborn calves was reported from sire-testing herds. In some cases the condition was so severe that the calves required euthanasia on humane grounds. Pedigree records showed that the calves were from the same sire and the inherited condition, epidermolysis bullosa (EB) was suspected.

EB is a family of mechanobullous disorders characterized predominantly by skin fragility resulting in blistering and defects of the skin and mucous membranes, among other tissues. EB affects the basement membrane (BM) zone of the skin and has been divided into three broad categories defined as simplex, dystrophic, and junctionalis (Fine *et al*, 2000; Uitto and Pulkkinen, 2001). Mutations in 10 genes have been identified as causes of EB (Uitto and Pulkkinen, 2001). The differentiation between different forms of EB requires ultrastructural examination of the site of cleavage, and allows a reduction in the number of candidate genes to be assessed.

In EB simplex, the skin separation occurs within the basal keratinocytes above the BM (Uitto and Pulkkinen, 2001). The simplex form of the disease in humans is known to be caused by mutations in four genes, namely keratin 5, keratin 14, integrin β4, and plectin (Human Gene Mutation Database 2003, http://archive.uwcm.ac.uk/uwcm/mg/hgmd0.htm; The Human Intermediate Filament Mutation Database 2002, http://www.interfil.org).

The keratins are structural proteins that form cytoplasmic networks within epithelial cells. Keratin intermediate filaments have a common structure that consists of a head and tail domain, with a central rod domain containing α-helical subsegments interspersed with linkers. Two regions, at either end of the central rod domain, are known to be highly conserved. Mutations in keratin genes have been identified as causing a number of human diseases (Irvine and McLean, 1999; Coulombe and Omary, 2002).

The frequency of reporting of affected calves suggested a possible recessive mode of inheritance or potentially a mosaic sire. Clinical presentation of the disease was assessed and electron microscopy (EM) examination carried out to define the disease as EB simplex, analogous to human EB simplex. Genetic analyses were undertaken to further characterize the disorder and to identify the underlying mutation.

Abbreviations: BM, basement membrane; EB, epidermolysis bullosa; EM, electron microscopy

Table I. Calves examined

Affected EB calf #	Calf sex	Age at examination (d)	Clinical examination	Post-mortem examination	Skin samples for EM
1	M	1	y	y	n
2	M	2	y	y	n
3	F	6	y	y	n
4	F	25	y	n	y

EB, epidermolysis bullosa; EM, electron microscopy; M, male; F, female; y, yes; n, no.

Results

Survey The survey showed that of 302 confirmed pregnancies, 187 calves were asymptomatic, 17 had clinical signs of EB, three were stillborn (cause unknown), two aborted, and the status of 93 was unknown. The latter category was most likely made up of male calves sent for slaughter soon after birth.

Clinical presentation The clinical reports of four of the affected calves (calves 1–4, Table I) were similar suggesting a single clinical phenotype (see Fig 1). The clinical findings from an affected bull calf (calf 1) were as follows: The calf, 8 h post-partum, was depressed and recumbent. He was bright immediately post-partum and had suckled. There were no apparent neurological defects. The skin on the lower limbs, base of the ears and base of the tail was reddened, edematous, and hairless. Small vesicular foci of approximately 2 mm in diameter were present over the edematous fetlock regions. Hair was also missing from contact areas such as the stifles, hocks, rump, and periorbital. There were horn defects on all feet, particularly at the heels where the horn had pulled away from the coronary band, and there was no horn at all on six of the dew claws (Fig 1*b*). Skin had sloughed off the dorsal aspect of the muzzle and mucosa had sloughed off both the nasolabium and areas within the oral cavity. There was bilateral corneal edema. The calf was euthanized on humane grounds and a post-mortem examination was carried out.

Post-mortem examination

Gross pathology Tissue samples from two calves (calves 1, 2), from the same sire, were examined. The findings for both calves appeared similar so a third affected calf (calf 3) was euthanized for a complete post-mortem examination. The findings of calf 3 are reported below. She was emaciated and dehydrated. There was extensive epidermal loss with ulceration and crusting of the skin, particularly distal to the fetlocks, and partial separation of bulbs and hooves from

Figure 1
Clinical features. (*a*) This clinical presentation was typical of the affected calves in this study. The calf was weak and required assistance to stand. Skin on the lower limbs, base of the tail, hips, shoulders, and muzzle was inflamed, edematous, and hairless. Hair was also missing from contact areas such as the stifles, hocks, rump, and periorbitally. Horn defects were evident on all feet, particularly at the heels, where the horn had pulled away from the coronary band. Mucosa had similarly sloughed off the nasolabium and lips. This calf also displayed bilateral keratitis. (*b*) Skin and hoof lesions of calf 3. All calves examined showed horn defects on all feet, particularly at the heels where the horn had pulled away from the coronary band. There was no horn on the dew claws (not shown). (*c*) Tongue and lower incisors of calf 3. All calves autopsied showed loss of mucosa from the oral cavity and tongue. Incisors were either unerupted or loose and splayed laterally. (*d*) Blister on normal appearing skin of calf 4 following irritation of normal skin. This effect, called Nikolsky's sign, is a diagnostic sign in a number of skin diseases in humans including different forms of epidermolysis bullosa (EB). This area, together with a grossly affected and unaffected area of skin, was sampled for electron microscopy (EM).

the coronary band. A yellow exudate was present over the skin of the ventral abdomen. There was severe mucopurulent crusting around the nostrils with mucocutaneous ulceration and the nasolabial epithelium lifted easily when handled. Hair could be epilated with ease from all areas of the body including those areas where the skin appeared normal. Multiple ulcers were present along the mucocutaneous lip border, the buccal aspect of the lips, the hard palate, and all aspects of the tongue (Fig 1*c*). The dorsum of the tongue was crusted with linear fissures and had a brown discoloration. The incisors were erupted but were loose and splayed laterally. The mandibular and maxillary molars were, however, only partially erupted and analogous to a still-born calf. There were no mucosal abnormalities in the esophagus or gastrointestinal tract.

Histopathology

Skin and mucous membranes Light microscopic examination of skin sections revealed clefts in the subbasilar (subepithelial) zone and the suprabasilar zones (see Fig 2*a*, *b*). In the former, this left a smooth BM lining on the floor of the ulcer and in the latter, an irregular partly cellular "tombstone"-like layer. In some sections including the right metatarsal, there was no epidermis remaining, leaving only a densely cellular dermis covered by necrotic neutrophils and fibrin. Numerous rod-shaped bacteria were also present in some sections.

Samples from the left metatarsal showed a clear region of subbasilar clefting and vascular, reactive dermis, but no significant inflammatory reaction. There were occasional apoptotic cells and some basal keratinocytes contained focal-condensed eosinophilic inclusions in their cytoplasm suggesting dyskeratosis (see Fig 2*c*). A hair follicle was similarly affected in this section. In adjacent areas, however, the intact epidermis was covered by a dense cellular crust and showed vacuolation of the basal layer of the epidermis (suprabasilar clefting). There was fibrinous exudate in spaces present in the superficial dermis and the BM appeared thickened. In a section from the nostril the basilar epidermal vacuolation was even more marked. Generally, sections of the oral mucosa were more damaged than the previously described skin sections and separation occurred at different levels. From the sections examined it was difficult to tell the exact site of cleavage because of secondary infection, inflammation, and trauma of the skin.

Other tissues The dorsum of the tongue showed pronounced separation of the dermis and epidermis with extensive bulla formation. The submucosa showed mixed lymphocytic-histiocytic-plasmacytic cellular infiltrations with congestion and fibrin coagulation in some vessels. The epithelium was acanthotic and parakeratotic. Numerous rod-shaped bacteria were also observed.

The corneal epithelium was intact laterally, vacuolated medially and absent in the central region. There was mixed lymphocytic–histiocytic and neutrophilic inflammation extending from the corneoscleral junction into the corneal stroma and through to the iridocilary angle.

The small intestine showed lymphoplasmocytic and eosinophilic infiltration of the mucosa. Mild diffuse vacuolation of hepatocytes was observed in liver sections. Occasional blood vessels in various internal organs showed leukocytosis and patchy intravascular fibrin accumulation similar to sections of the tongue. The following diagnoses were made:

1. Mechanobullous dermatitis and stomatitis with suprabasilar clefting;
2. Keratitis with ulceration and bulla formation.

The level of clefting was variable but it appeared from the most intact sections of the skin that cleavage was at the BM and thus indicating the condition was analogous to the junctional form of EB demonstrated in humans.

EM Thin sectioning was performed on samples from normal appearing, irritated, and affected skin collected from an affected calf (calf 4). The site of clefting between the epidermis and the dermis was best visualized in the grossly normal-appearing sections of the affected calf. Here clefting appeared to be above the BM as intact keratinocytes and cellular debris, presumably from necrotic keratinocytes,

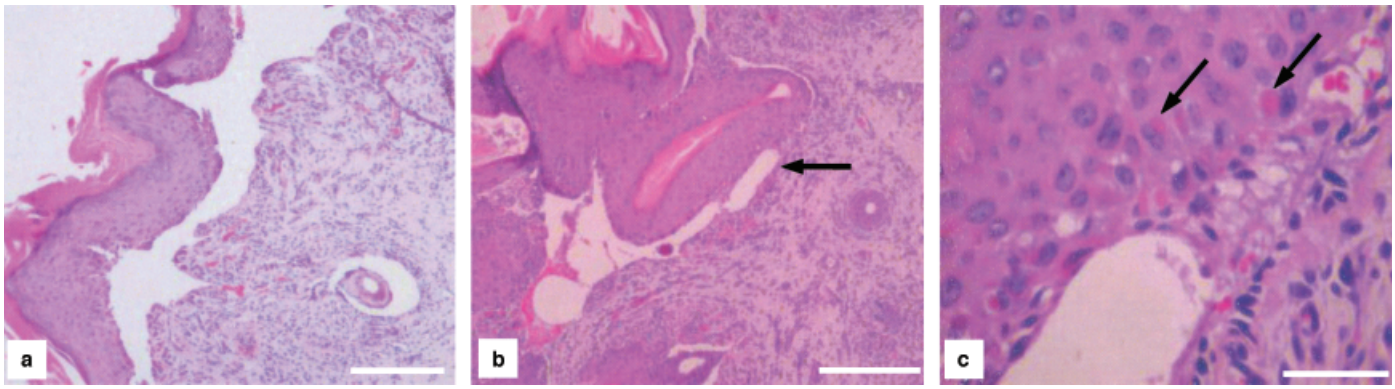

Figure 2
Light microscopy of affected tissue. (*a*) Light micrograph of a hematoxylin and eosin-stained section from the left metatarsal region of calf 3 showing dermo-epidermal cleft formation. *Scale bar*, 200 μm. (*b*) A second low power view of the dermo-epidermal cleft shown in (*a*). Here a "bridge" between the separating epidermis and dermis is evident. A high power view of one area of the cleft (*black arrow*) is shown in (*c*). *Scale bar*, 300 μm. (*c*) High power view of dermo-epidermal cleavage from the left metatarsal region of calf 3. Clefting is visible along the dermo-epidermal junction, but the exact site of separation is indistinct. Eosinophilic clumps can be seen in the epithelial cytoplasm (*black arrows*), which are thought to correlate with the tonofilament clumping or possibly the vesicles observed with electron microscopy (EM). *Scale bar*, 40 μm.

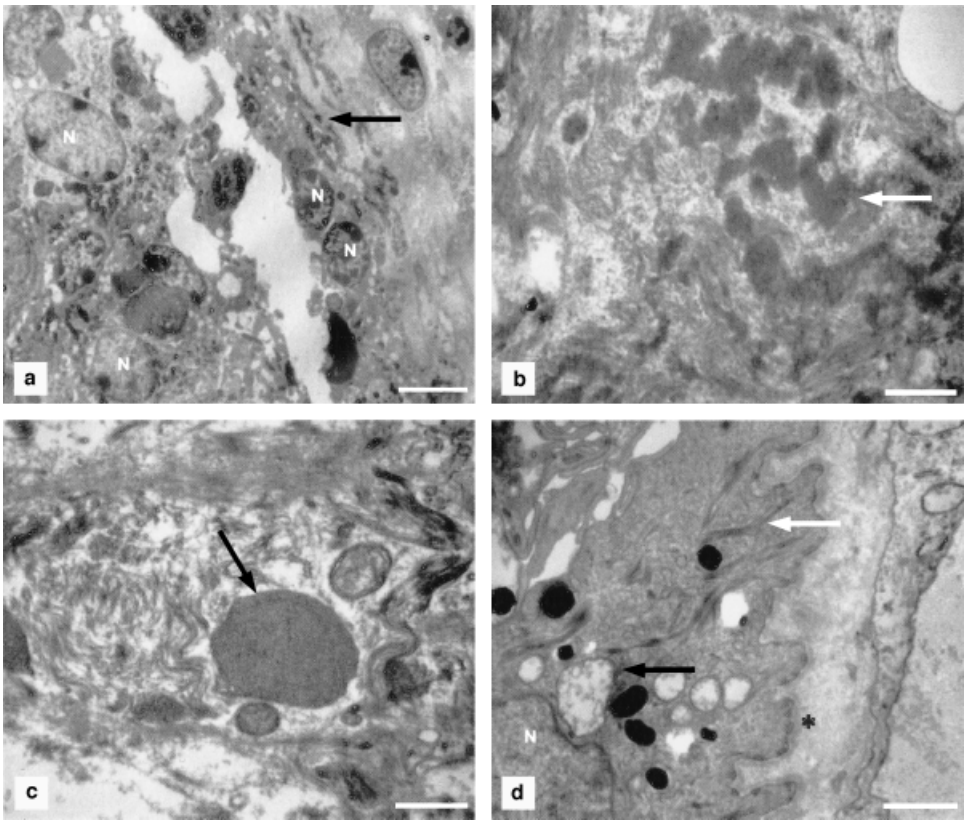

Figure 3
Electron micrograph of tissue samples. (*a*) This electron micrograph from normal-appearing skin of calf 4 shows the dermo-epidermal cleft created by lysis of the basal keratinocytes. Keratinocyte nuclei (N) and cellular debris are visible between the cleft and the basement membrane (BM) (*solid black arrow*). This bovine form of epidermolysis bullosa (EB) is analogous to the human simplex form: *Scale bar*, 5 μm. (*b*) An electron micrograph from normal-appearing skin of calf 4 shows clumping of tonofilaments (*white arrow*) in a basal keratinocyte. This is a characteristic finding in the simplex form of EB along with lysis of the basal keratinocytes (see (*a*). *Scale bar*, 0.5 μm. (*c*) A large vesicle with granular contents was seen in a few keratinocytes (calf 4) (*black arrow*) in irritated skin samples from an EB calf. It was found among abnormal-appearing tonofilaments and the contents may represent remnants of the tonofilaments. Similar structures have been described in the Dowling–Meara form of EB simplex in humans where they were described as condensed tonofilaments and in familial acantholysis in cattle where they were described large eosinophilic intracytoplasmic bodies. *Scale bar*, 0.5 μm. (*d*) Electron micrograph of a skin sample from normal (*unaffected*) calf. Normal architecture of a basal keratinocyte including tonofilaments (*white arrow*), a hemidesmosome at the BM (*) and mitochondria (*black arrow*). *Scale Bar*, 1 μm.

were seen between the clefts and the intact BM of the epidermis (Fig 3*a*). Clumping of tonofilaments was seen in a number of basal cells (Fig 3*b*) as seen in human cases of EB (Anton-Lamprecht and Schnyder, 1982; Ishida-Yamamoto *et al*, 1991). Occasionally intracytoplasmic vesicles were observed containing a granular substance that was believed to represent a mass of condensed tonofilaments (Jaunzems *et al*, 1997) (Fig 3*c*). These may represent the focal-condensed eosinophilic inclusions (Fig 2*c*). This vesicular structure, however, is only 0.5 μm in size and the esinophilic inclusions appear to be between 4 and 5 μm suggesting clumped tonofilaments rather than the vesicle may in fact be appearing as eosinophilic inclusions. In comparison there was no tonofilament clumping or cleft formation in the normal calf skin sections (Fig 3*d*).

The site of clefting appeared to be within the basal keratinocytes and this along with the tonofilament clumping suggested that the affected calf had a condition analogous to EB simplex in humans rather than the junctional form of the disease.

Comparative mapping Of the four genes where mutations have been shown to cause EB simplex, mutations in keratin 5 and keratin 14 are responsible for the majority of cases (Human Gene Mutation Database 2003, http://archive.uwcm.ac.uk/uwcm/mg/hgmd0.htm, The Human Intermediate Filament Mutation Database 2002, http://www.interfil.org).

Using comparative bovine and human genome maps, the putative positions of the keratin 5 and keratin 14 genes were determined on the bovine genome (ArkDB, http://bos.cvm.tamu.edu/bovarkdb.html). Human keratin 5 is located on human chromosome 12, the syntenic region on bovine chromosome 5. Human keratin 14 is located on human chromosome 17, the syntenic region on bovine chromosome 19. Bovine microsatellite markers close to these genes were determined and used to genotype the sire, six of his affected calves and their dams. The markers used were BP1, close to keratin 5 and IDVGA44, close to keratin 14. There was no significant association of IDVGA44 to the disease phenotype; however, one allele of the marker close to keratin 5 was present in all affected animals (Fig 4).

Bovine keratin 5 gene analysis The bovine keratin 5 sequence was established as follows. A bovine 2 kb fragment of 5′ UTR and the start of the coding region were obtained (Genbank accession number Z32746). Sequence of human keratin 5 (Genbank accession number AF274874) was used to search the Genbank database as well as the

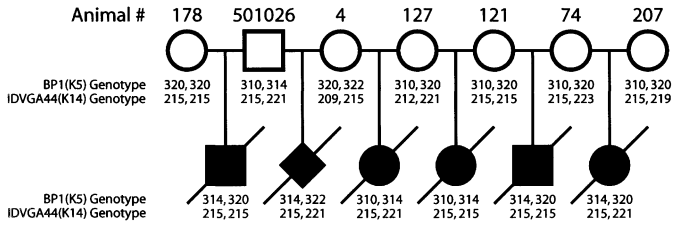

Figure 4
A pedigree diagram giving genotypes (alleles) for each microsatellite marker. This demonstrates that the BP1 (keratin 5) allele 314 is inherited from the sire in all affected animals. Squares represent male animals, circles represent female animals, and a diamond represents an animal of unknown gender. Filled shapes represent affected animals and lines through the shape represent deceased animals.

ViaLactia Biosciences Ltd (Auckland, New Zealand) in-house bovine EST library for sequences corresponding to bovine keratin 5. This revealed five sequences (Genbank accession numbers, BE589787, BE478404, BE588471, BG690177, BE479944) from the public database and seven sequences from the ViaLactia database. All of these sequences were assembled to give a putative cDNA sequence. This sequence data was used to design primers (K5F1, K5R4) to amplify the full-length bovine keratin 5 genomic fragment, 5815 bp of gene sequence, 113 bp 5′ UTR, and 66 bp of 3′ UTR (Genbank accession number AY740402). This genomic region was amplified and sequenced using DNA extracted from blood from four animals: a normal, the sire, an affected calf, and the calf's dam. Comparison of the sequence revealed one base change in one allele of the affected calf, a G-to-A substitution at position 4164 of the genomic sequence (position 4051 from the ATG start). This results in a codon change from GAG (Glutamic acid, E, acidic) to AAG (Lysine, K, basic) at position 478 of the amino acid sequence (final E of the KLLEGE domain). The dam was homozygous G normal whereas the sire showed some evidence that the A allele was present but not at the level expected for true heterozygosity (mosaicism). Exon–intron boundaries were identified in the bovine sequence by comparison with the human sequence. The putative cDNA sequence derived from the genomic sequence was 1806 bp in length, the mutation occurring at position 1432, and encodes a protein of 601 amino acids. Comparison with the coding regions of the human and bovine keratin 5 gene showed approximately 90% homology.

A 348 bp region surrounding the mutation was then sequenced in six affected calves, their dams, 50 unaffected animals derived from the investigated sire, 10 dams of unaffected calves, and the parents of the sire. An example of the sequence trace in a normal and affected animal is shown in Fig 5. All affected calves were heterozygous for the G-to-A substitution and all unaffected calves and all dams were homozygous G normals. The parents of the sire were also homozygous G normal.

Protein sequences of human (Genbank accession number AF274874) and bovine keratin 5 were compared and revealed 95% identity and 96% similarity (Seqweb Accelrys, San Diego, California). In addition to the amino acid change, the bovine keratin 5 protein has an additional 12 amino acids adding additional GGGL repeats to the region near the COOH terminal of the protein. The affect of this insertion on the function of bovine keratin 5 is not known. Comparison with mouse protein sequence (Genbank accession number AAL16774) showed a much lower degree of similarity although the KLLEGE motif is conserved.

Mosaicism Digestion of the 173 bp mutation containing PCR product with BseR1 (New England Biolabs, Beverly, Massachusetts) revealed that both the blood and semen samples of the sire contained the mutant allele, although in lower proportions than that observed in the affected heterozygous offspring (i.e., mosaic) (Fig 6). An estimate of the frequency of the mutant allele in the sire samples was carried out using Quantity One software (BioRad, Hercules, California), assuming equivalent efficiency of amplification of each allele and compensating for altered binding of ethidium bromide by differently sized DNA fragments. The sire blood and semen samples have the mutant allele present at approximately 20% frequency, showing that the sire is mosaic in these tissues.

This data fits with the unaffected status of the sire and the lower-than-expected frequency of affected offspring assuming that the mutation is autosomal dominant in effect.

Discussion

After an initial clinical report, a more thorough analysis revealed a potentially familial form of EB in calves from a single sire. Clinical examination of the affected calves revealed fragility in numerous tissues including skin and oral

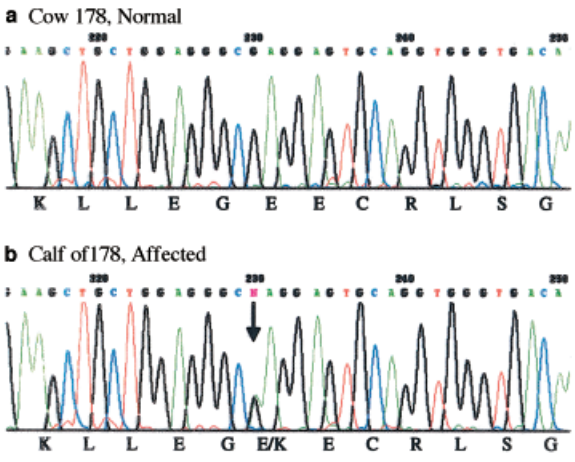

Figure 5
Sample of an electropherogram from an affected calf and its normal dam. The electropherograms show the presence of both the G and A nucleotides in the affected animal. Translation of the region is shown below. (*a*) Sequence of the mutation region of keratin 5 for cow 178 who displays a normal phenotype. (*b*) Sequence of the mutation region of keratin 5 for the calf of cow 178 who displayed an affected phenotype. The arrow shows the heterozygous sequence resulting in a codon change E478K.

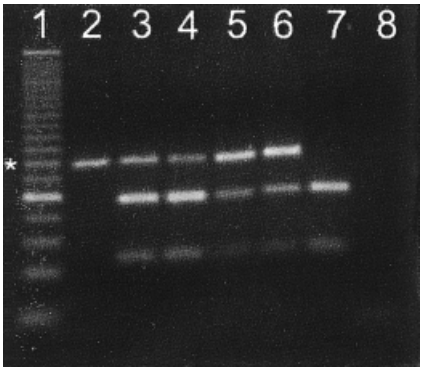

Figure 6
Agarose gel of restriction enzyme digest of the 173 bp single nucleotide polymorphism (SNP) containing PCR product. *Lane* 1, 25 bp ladder (Invitrogen, Carlsbad, California), *lane* 2, uncut sample; *lane* 3, sire blood sample; *lane* 4, sire semen sample; *lane* 5, calf of cow 207 (affected); *lane* 6, calf of cow 74 (*affected*), *lane* 7, cow 207 (*unaffected*); *lane* 8, no template control. *Note reduced intensity of the 173 bp band in the mosaic sire.

mucosa. Light microscopy analysis of the skin identified the site of cleavage to be around the BM. EM further identified the cleavage site as being above the BM leading to a diagnosis, and it was subsequently refined to EB simplex (analogous to human EB simplex), likely to involve a keratin. The inclusions observed in the basal keratinocytes are thought to represent clumping of the intermediate filaments. Transgenic mice expressing a mutant keratin showed evidence of keratin aggregates and lysis of basal cells (Vassar *et al*, 1991).

Keratins are building blocks for a filamentous scaffold within epithelial cells that allow them to withstand mechanical and non-mechanical stress. They are divided into two subtypes, type I (e.g., keratin 14) and type II (e.g., keratin 5), which combine as heterodimers to form intermediate filaments or tonofilaments. They have a central α-helical rod domain (with subsegments 1A, 1B, 2A, and 2B) flanked by non-helical head and tail domains (Coulombe and Omary, 2002). Within the 2B rod domain is a KLLEGE motif that is highly conserved and has been shown to be required for lateral alignment of keratins (Wilson *et al*, 1992). Keratin 5 was identified as the most likely candidate through linkage and the bovine keratin 5 sequence determined. Sequence analysis of normal and affected pedigree members revealed a single-base mutation.

This nucleotide substitution is located in exon 7 at the end of the 2B rod domain, and results in an amino acid change of E-to-K in the final E of the KLLEGE motif. It is possible that the amino acid change (acidic to basic) alters the formation of keratin heterodimers and intermediate filaments, leading to keratinocyte fragility and separation of the epidermis.

Given that one mutant allele is sufficient to cause disease, the mutation is clearly dominant; however, the sire was unaffected. In addition, we would have expected to see higher numbers of affected offspring derived from this sire (even taking into account observer effects) that lead us to consider mosaicism. The antecedents of the sire do not have the mutation so it must have arisen spontaneously during the sire's development. The possibility of reduced penetrance was excluded through an analysis of 50 unaffected animals of the sire, none of which carried the mutant allele. Germline mosaicism has been reported in other keratin disorders, for example, keratin 10 causing epidermolytic hyperkeratosis (Paller *et al*, 1994; Rothnagel, 1998). Germline mosaicism has also been observed in a human case of EB. In this case a laminin B3 mutation caused junctional EB in offspring of clinically unaffected parents (Cserhalmi-Freidman *et al*, 2002).

Indeed DNA analysis revealed a lower frequency of the mutant allele in samples obtained from blood and semen indicating that this sire was mosaic. This explains both the sire being clinically unaffected and the lower than expected number of affected offspring. Perhaps surprisingly, the same mutation found in this sire has been reported in human cases of EB simplex. The human mutation was described as *de novo* in two patients with EB simplex and was autosomally dominant (Stephens *et al*, 1997).

The progeny of this sire are an ideal alternative model for the human disease (in addition to the mouse models), which could be used to further study the genotype/phenotype relationship or for studies of gene therapy. Semen from the sire animal has been collected and stored, and could be used for breeding animals to use as EB models.

Materials and Methods

All animal sampling was approved by the local Ethics Committee in accordance with Helsinki Guidelines.

Survey Records of the breeding company indicated that a maximum of 750 artificial inseminations occurred with semen from the carrier sire that would typically translate to around 450 pregnancies. All farmers that used semen from this bull were surveyed and from the respondents 302 pregnancies and their outcomes were reported.

Sampling Clinical examinations were carried out by veterinary practitioners on three affected calves (1, 2, and 4) (see Table I). Post-mortem examinations were carried out on three calves (1–3) and routine visceral organs, sections of skin, tongue, and oral mucosa were fixed in 10% buffered formalin, embedded in paraffin wax, sectioned at 5 μm, and stained with hematoxylin and eosin using routine processing methods. The asymptomatic sire and 10 unaffected calves were also given a thorough clinical examination.

EM Samples of normal and grossly affected skin were taken from an affected calf and a sample of skin irritated (to induce a bullous lesion) immediately prior to euthanasia. Random normal calf skin was collected from an abattoir. All samples were placed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). One millimeter slices were taken, washed in phosphate buffer, fixed in 1% osmium tetroxide, stained with aqueous uranyl acetate, dehydrated through a graded series of ethanol and propylene oxide, and embedded in Procure 812m resin (ProSciTech, Kirwan, Queensland, Australia). Thin sections were then collected on copper grids, stained with aqueous uranyl acetate and lead citrate, and examined on a Hitachi H-7000 transmission electron microscope (Hitachi Ltd, Tokyo, Japan). Images were captured using a Megaview III digital camera (Software Imaging System GmbH, Münster, Germany).

DNA extraction DNA was extracted from 10 mL of blood using the lysis of blood cells protocol (Sambrook and Russell, 2001).

Pedigree analysis Microsatellite markers were typed using the following conditions; PCR: 1 $\times$ buffer, 2.5 mM $MgCl_2$, 0.16 mM dNTP's, 0.4 μM each primer, 25 ng DNA template, and 0.9 U AmpliTaq Gold DNA Polymerase (Applied Biosystems, Foster City, California). One primer per primer pair was 5′ labelled with a fluorescent dye (Applied Biosystems). The cycling protocol was 94°C for 10 min; five cycles of 94°C for 30 s, 59°C for 45 s (decreasing 1°C every cycle), 72°C for 30 s, 76°C for 30 s; 30 cycles of 94°C for 30 s, 54°C for 45 s, 72°C for 30 s, 76°C for 30 s; one cycle at 72°C for 1 h; one cycle at 25°C for 2 h. Primer sequences were as specified in Table II.

Table II. Primers for pedigree analysis

Name	Sequence	Label	Marker
BP1 for	5′AAAATCCCTTCATAAC-AGTGCC3′	6FAM	BP1
BP1 rev	5′CATCGTGAATTCCAG-GGTTC3′	—	BP1
IDVGA44 for	5′GGGAGAATGGATGG-AACCAAAT3′	6FAM	IDVGA44
IDVGA44 rev	5′TTCGAAGACGGGCA-GACAGG3′	—	IDVGA44

Table III. Primers used for keratin 5 analysis

Name	Sequence
K1	5′CGCCCAAACCAGGTCTAGAG3′
K2	5′ACTTGGCTTGAGACACCACCTAG3′
K3	5′AGAAAGAGACCCCTTAGGCCC3′
K4	5′AGCTCTACTTTATCAAACGTGAGGC3′
K5	5′AAGCAGGACATGGCACGTC3′

A portion of the diluted samples were mixed with GeneScan-400HD [ROX] size standard and HiDi Formamide (Applied Biosystems). This mix was denatured at 95°C for 5 min and held on ice for 2 min before running on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). Data analysis was carried out using GeneScan Analysis software (Applied Biosystems), with genotypes scored using Genotyper software (Applied Biosystems).

Isolation and analysis of bovine keratin 5 A 6 kb PCR product incorporating the majority of the bovine keratin 5 gene was generated using primers K1 and K2 (see Table III) and the Expand 20 kb^{PLUS} PCR System (Roche, Penzberg, Germany), according to the manufacturer's instructions.

Amplification of a 348 bp fragment surrounding the G–A (Nt 4051) single nucleotide polymorphism was undertaken using primers K3 and K4 (see Table III) and the following reaction conditions; 1 × Gold PCR buffer, 2.5 mM $MgCl_2$, 0.2 mM each dNTP, 0.4 μM each primer, and 2.5 U AmpliTaq Gold DNA Polymerase (Applied Biosystems). The cycling protocol was 94°C for 5 min, then 35 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 30 s, and a final extension at 72°C for 2 min.

All PCR products were purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) followed by sequencing on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems) to specifically detect the G–A (Nt 4051) mutation.

To determine the approximate level of mosaicism, PCR around the mutation was carried out using primers K4 and K5 (see Table III), with the same protocol as used for the 348 bp fragment except that the annealing temperature was 57°C. Following purification of the 173 bp PCR product, a fraction was digested with the restriction enzyme BseR1 (New England Biolabs). The recognition site of BseR1 is GAGGAG, the site present in the normal allele only (site changed to AAGGAG in the mutant allele). Digestion of the 173 bp fragment results in two fragments of size 118 and 55 bp in normal animals, whereas heterozygous affected animals retain the 173 bp fragment. Samples were separated on a 3.5% NuSieve GTG agarose TAE gel (Cambrex Bioscience, Rockland, Maine).

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Appendix: Electronic-database information

The URLs for data presented herein are as follows

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